Chronic exposure to benzo(a)pyrene-coupled nanoparticles worsens inflammation in HDM-induced asthma mouse model

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MATERIAL AND METHODS

Carbon black nanoparticle coating

Carbon nanoparticles were kindly provided by Dr Sophie Lanone from Institut Mondor de Recherche Biomédicale and previously characterized by Belade *et al*. [1] A part of these nanoparticles was coupled to B(a)P. A solution of B(a)P (Sigma-Aldrich, Lyon, France) at a concentration of 5.55 mg/ml was prepared with dichloromethane (Sigma Aldrich) as solvent. A mass of 1 g of carbon nanoparticles was mixed with 10 ml of this solution, and the solvent was completely evaporated at room temperature in a rotary evaporator (about 3 hours for total evaporation). After recovery of the coated nanoparticles, a control of the amount of B(a)P remaining on the walls of the flask of the rotary evaporator was carried out by GC-MS analysis (GC Perkin Elmer 680, MS Perkin Elmer 600C). The B(a)P not present on the walls is supposed to cover the particles; direct analysis of the B(a)P adsorbed on the nanoparticles is indeed made difficult by its non-total extraction on this support. The final B(a)P concentration on the nanoparticles was 55.5 mg/g. Nanoparticles were stored in brown vial at -20°C.

Animals

C57BL/6 J Female mice (6 weeks old) were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animal study was approved by the local investigational review board (N° APAFIS#7874-2016070417344442v3) in an accredited establishment at the Institut Pasteur de Lille according to governmental guidelines N°86/609/CEE and the European directive 2010/63/EU. Mice were housed under standard conditions in a pathogen-free facility.

Experimental protocol

After isoflurane gas anesthesia, mice were sensitized intranasally by 25 µg of HDM (Stallergenes Greer, Boston, USA) for 3 consecutive days with or without the presence of 18 µg of uncoated or B(a)P-coupled nanoparticles corresponding to 1 µg of B(a)P. Twelve days after, mice were challenged intranasally in the same conditions 3 days a week during 4 weeks. Mice were euthanized 24 hours after the last intranasal by injection of Euthasol lethal dose. The model for chronic exposure is shown in figure S1A. BAL fluids and cells, blood samples and lung tissues were collected for further analyses.

BAL fluid and cell collection

A total volume of 1 mL of ice-cold phosphate buffer saline (PBS) (Fisher Scientific, Courtaboeuf, France) was used to wash the lungs. Total leukocyte numbers were counted, cytocentrifuged (Shandon cytospin 4; ThermoFisher Scientific, Waltham, USA) and stained with May-Grünwald Giemsa (Microm Microtech, Brignais, France). Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard hematological procedures and at least 300 cells were counted under x400 magnification.

Serum antibodies measurement

HDM-specific IgE and IgG_1 levels were determined by enzyme linked immunosorbent assay (ELISA) using 100 µg/ml HDM (Stallergenes Greer) coated in wells. After incubation with diluted serum, specific IgE and IgG_1 antibodies were detected by biotinylated goat anti-IgE (Gentaure) or IgG_1 (ThermoFisher scientific). Binding of Streptavidin-Horse Radish Peroxidase was revealed by TMB substrate solution (Sigma-Aldrich, St Louis, USA) and the OD value at 450 nm was determined.

Airway hyperresponsiveness measurement

Airway hyperresponsiveness (AHR) was assessed 72 hours after the last instillation using flexiVent invasive method (SCIREQ, Montreal, Quebec, Canada). Mice were anesthetized (5 ml/kg body weight of 10% medetomidine (Pfizer) and 10% ketamine (Merial)) and immediately intubated with an 18-gauge catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H2O was applied. Mice were exposed to nebulized PBS followed by increasing concentrations of nebulized methacholine (0-100mg/ml in PBS) (Sigma-Aldrich, St Louis, MO, USA) using an ultrasonic nebulizer (Aeroneb, Aerogen, Galway, Ireland). For each dose, 10 cycles of nebulization and measurements were performed. Nebulization was done during the first cycle and consisted of 20 puffs per 10 s, with each puff of aerosol delivery lasting 10 ms. For each cycle, measurements were obtained for 15 s followed by ventilation for 5s. Baseline lung resistance (Rrs) was restored before administration of the subsequent doses of methacholine. Only resistance values corresponding to COD values > 0.95 were kept. For each dose, the maximum resistance value measured was taken.

Histology on lung tissue sections

Left lung was fixed in Antigenfix (Diapath, Microm Microtech, France) during 4 hours at room temperature followed by alcohol (30-100%) and Diasolv (Microm Microtech) baths for dehydration. After paraffin inclusion, Paraffin-embedded lungs were sliced (5 µm) with a Microtom (Microm HM355S Thermoscientific). Sections were stained using periodic acid–Schiff (Microm Microtech) for mucus production and Hematoxylin & Eosin (Diapath, Microm Microtech) for cellular infiltration. Images were acquired on an AxioPlan2 (Zeiss) light microscope with Zen Pro software.

RNA Isolation and Quantitative RT-PCR

RNA was extracted from lung with a nucleospin RNA mini kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. Extracted RNA was reverse-transcribed with the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. The real-time RT-qPCR was performed in Prime time assay PCR Master Mix (Integrated DNA technologies, Leuven, Belgium) using QuanStudio 12K Flex Real-Time PCR System (Thermofisher Scientific). The quantitative RT-PCR cycling were: one cycle at 95°c for 3 min followed by 45 cycles composed by 95°C for 5 s and 60°C for 30 s. Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing the PCR cycle thresholds (Ct) for the gene of interest and *rplp0* (ΔCt) and ΔCt values for treated and control groups ($\Delta\Delta$Ct). The primers (Integrated DNA technologies) used for RT-qPCR are shown in Table S1. Ribosomal Protein Lateral Stalk Subunit P0 gene (*Rplp0*) was used as a reference gene in order to normalize the transcript levels.

Flow Cytometry experiments

Circulation was washed by heart perfusion with 10 ml of PBS. Total lungs were digested by 1 mg/mL type IV collagenase (Life Technologies, Courtaboeuf, France) in RPMI (Life Technologies) for 30 min at 37°C. Cells were washed with PBS with 2% fetal bovine serum (FBS) (Eurobio scientific, Courtaboeuf, France) and centrifuged at 2000 rpm for 15 min on a Percoll density gradient (D = 1075 g/mL; Amersham Pharmacia Biotech, Piscataway, NJ). Erythrocytes were lysed in lysis buffer (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, and 0.5 mmol/L EDTA). Cells were washed with PBS/FBS 2% and filtered on a 100-µm-pore membrane. Alive total lung cells were counted after Trypan Blue staining. Cells were incubated with viability marker (Zombie aqua™ Fixable Viability Kit, Biolegend, Ozyme, Saint-Cyr-L'Ecole, France) and incubated for 30 min at 4°C with conjugated antibodies as presented in supplemental data Table S2 and S3. The cells were washed and resuspended in PBS/Fetal Bovine Serum 2% and analyzed by flow cytometry (LSRFortessa®, BD Biosciences, San Diego, CA, USA) with the BD FACSDiva™ Software.

Statistical analysis

The data are presented as median min. to max. and plots. All experiments were carried out at least twice. The comparisons between groups were performed using Kruskal-Wallis nonparametric test with Dunns post-hoc test in GraphPad Prism between HDM groups or between HDM and controls groups (GraphPad software, Inc., La Jolla, CA, USA). *p*-value <0.05 were considered as statistically significant.

SUPPLEMENTARY RESULTS

Nanoparticles coupled to B(a)P activate AhR signaling pathway

We first aimed at characterizing the molecular pathway triggered by NPs coupled to B(a)P. B(a)P is known to activate AhR signaling pathway and especially cytochrome transcription.[S2] Therefore, we evaluated the expression of three *cyp* genes *cyp1a1*, *cyp1a2* and *cyp1b1* in total lung extract. NP-B(a)P significantly increased *cyp1a1* but not *cyp1a2* and *cyp1b1* expression compared to uncoated NPs (NP-Ø). Interestingly, chronic exposure to HDM alone did not induce *cyp* genes transcription (Figure S4).

The co-exposure of mice with NP-B(a)P and HDM (HDM+NP-B(a)P) also increased *cyp1a1* but not *cyp1a2* and *cyp1b1* expression compared to NP-Ø with HDM (HDM+NP-Ø). However, the presence of HDM tended to decrease *cyp1a1* expression compared to NP-B(a)P alone (Figure S4). These results show that B(a)P fixed on the NPs is able to activate AhR signaling pathway.

SUPPLEMENTARY REFERENCES

1. Belade, E. *et al.* A comparative transmission electron microscopy study of titanium dioxide and carbon black nanoparticles uptake in human lung epithelial and fibroblast cell lines. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA* **26**, 57–66 (2012).
2. Stockinger B, Di Meglio P, Gialitakis M, Duarte JH. The aryl hydrocarbon receptor: multitasking in the immune system. Annu Rev Immunol. 2014;32:403-32.

SUPPLEMENTARY TABLES

Supp. Tab. S1: Prime time assay used for the quantitative PCR

Target	Catalogs	Target	Catalogs
cyp1a1	Mm.PT.58.32054918	*il-10*	Mm.PT.58.13531087
cyp1a2	Mm.PT.58.18171461	*il-13*	Mm.PT.58.31366752
cyp1b1	Mm.PT.58.43705524	*il-17*	Mm.PT.58.6531092
ifn-γ	Mm.PT.58.41769240	*muc5ac*	Mm.PT.58.42279692
il-4	Mm.PT.58.7882098	*muc5b*	Mm.PT.58.30457752
il-5	Mm.PT.58.41498972	*rplp0*	Mm.PT.58.43894205

Supp. Tab. S2: Flow cytometry antibodies for T cells staining

Protein Target	Conjugate	Species Target	Company
TCRβ	APC-Vio770	Mouse	Miltenyi Biotech

TCRγ/δ	Vioblue	Mouse	
CD4	PE-Vio770	Mouse	
CD25	APC	Mouse	
CD8a	BV605	Mouse	Ozyme (Biolegend)
CD5	PerCP-Cy5.5	Mouse	
NK1.1	FITC	Mouse	
CD45	PE-Cy5	Mouse	

Supp. Tab. S3: Flow cytometry antibodies for antigen presenting cells staining

Protein Target	Conjugate	Species Target	Company
LY6C	BV605	Mouse	BD Biosciences
CD64	BV786	Mouse	
CD103	PerCP-Cy5.5	Mouse	
CD86	AF700	Mouse	
CD11c	PE-Cy7	Mouse	
CD11b	V450	Mouse	
CD45	PE-Cy5	Mouse	
Siglec F	PE-CF594	Mouse	
F4/80	PE	Mouse	Miltenyi Biotech
CMHII	FITC	Mouse	
CCR2	BV650	Mouse	Ozyme (Biolegend)
CD206	APC	Mouse	
LY6G	APC-Cy7	Mouse	